2D NMR in structural studies of natural compounds

model study:

(O-antigen of Citrobacter freundii O22)
Sugar analyzer:

- **Rha** (2 eq, 6d)
- **Gal** (4 eq)
- **Man** (3 eq)
- **Unknown**

**NMR $^{31}$P**
- Empty

**NMR $^1$H**
- 4 anomers
- 2 -CH$_3$ (C6)
- -CH$_2$- (ring)
- HDO

**NMR $^{13}$C BB**
- 4 anomers
- no furanoses
- sugar ring
- substituted -CH(OH)-
- 2 -CH$_2$OH (C6)
- 2 -CH$_3$ (C6)
### Experiments

<table>
<thead>
<tr>
<th>Question</th>
<th>1H 1D NMR</th>
<th>proton spectrum - general information</th>
</tr>
</thead>
<tbody>
<tr>
<td>NMR experiment selection</td>
<td>1H HD diff</td>
<td>selective homonuclear decoupling - revealing of signals of neighboring protons</td>
</tr>
<tr>
<td>Acquisition</td>
<td>13C, 31P, ... BB</td>
<td>broad-band proton decoupled spectra - additional information, “fingerprint”, substitution positions</td>
</tr>
<tr>
<td>Interpretation</td>
<td>13C DEPT, APT, 15N INEPT</td>
<td>edited selective polarization transfer - revealing carbon and nitrogen protonation and sensitivity gain</td>
</tr>
<tr>
<td>Answer</td>
<td>13C Gated</td>
<td>undecoupled carbon spectrum - heteronuclear coupling constants measurement</td>
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<tr>
<td>1H NOE diff</td>
<td>nuclear Overhauser effect measurement - proton-proton spatial contacts</td>
<td></td>
</tr>
</tbody>
</table>

| COSY, COSY-45 | homonuclear spin correlation - proton spectrum assignment |
| COSY n-RCT | relayed coherence transfer in COSY - proton spectrum assignment |
| DQF COSY | double quantum filtered COSY - assignment of proximal signals |
| TOCSY | total homonuclear correlation - distinguishing of proton spin systems |
| NOESY, ROESY | homonuclear spatial correlation - revealing of residue sequence and conformation studies |
| DOSY | diffusion ordered spectroscopy - separation of a spectrum into component subspectra |

| 1H, 13C HSQC | proton-carbon spin correlation - carbon spectrum assignment |
| 1H, 31P HSQC | proton-phosphorus spin correlation - phosphate groups localization |
| 1H, 13C HMBC, 1H, 15N HMBC | multiple-bond heteronuclear spin correlation - revealing modifier attachment pattern and residue sequence |
| HSQC Relay | relayed heteronuclear spin correlation - tracking neighboring carbons |
| HSQC-TOCSY | total heteronuclear correlation - distinguishing of residue spin systems |
$\{^1H, ^1H\}$ COSY

$^3J_{H_1-H_2} < 3$ Hz for Gal
$\Rightarrow \alpha$-Gal

$J < 5$ Hz at H2

$C_5$ at 74.8 $\Rightarrow$ not $\alpha$-Gal

$J \sim 10$ Hz at H2

remaining $\delta H_6$

$\delta H_3, \delta H_6$
\{^{1}H,^{13}C\} HSQC

C5 in free sugar:
αGal 71.7
αMan 74.2
βMan 77.4

if unsubstituted, identified as α-3,6ddXylHex

from HMBC
H1/C3, H2/C3
from HMBC
H2/C4
from HMBC
H1/C5

ambiguous
abnormally low-field

αMan C4
C5 α
\{^{1}H, ^{13}C\} HSQC-TOCSY

H1/C2, H1/C3

see HSQC
by exclusion:

\[
\text{ddXylHex(1}\rightarrow?\text{)Man}
\]

\[
\text{Gal(1}\rightarrow?\text{)Man}
\]

\[
\text{Rha(1}\rightarrow3\text{)Gal}
\]

\[
\text{Man(1}\rightarrow4\text{)Rha}
\]
\[ {^{1}H, ^{13}C} \text{HMBC} \]

- Find C5
- Find H5
- Find C3, C5
- H1/C5
- H1/C3
- H1/C4
- ddXylHex at C3
- Gal at C2
- H2/C1
- H2/C4
- H4/C3
- H4/C5
- H2/C3
- H4/C1
- H6/C4
- H6/C5
- H6/C4
- H6/C5
- H6/C4
<table>
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<td>α-Rhap</td>
<td>103.3</td>
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<td>α-Manp</td>
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<td>79.0</td>
<td>67.7</td>
<td>74.8</td>
<td>62.0</td>
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<tr>
<td>→4)α-Rhap</td>
<td>8.1</td>
<td>-0.2</td>
<td>-0.7</td>
<td>9.5</td>
<td>-0.1</td>
<td>0.6</td>
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<td>α-3,6ddXylp</td>
<td>9.3</td>
<td>0.7</td>
<td>0.2</td>
<td>0.3</td>
<td>1.0</td>
<td>0.4</td>
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<tr>
<td>→3)α-Galp</td>
<td>9.3</td>
<td>-0.2</td>
<td>8.2</td>
<td>-0.1</td>
<td>1.2</td>
<td>0.4</td>
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<tr>
<td>→2,3)α-Manp</td>
<td>5.8</td>
<td>9.4</td>
<td>7.5</td>
<td>-0.5</td>
<td>0.6</td>
<td>-0.3</td>
</tr>
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</table>

**NMR $^{13}$C BB**

![NMR spectrum](image)
### Absolute configurations

<table>
<thead>
<tr>
<th>Residue Pair</th>
<th>Carbon</th>
<th>Theory</th>
<th>Experiment</th>
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<tbody>
<tr>
<td>Man → 4αRha</td>
<td>C-4</td>
<td>DD 7.6</td>
<td>9.2</td>
</tr>
<tr>
<td>Rha → 3αGal</td>
<td>C-3</td>
<td>DD 3.9</td>
<td>8.1</td>
</tr>
<tr>
<td>Gal → 2αMan</td>
<td>C-2</td>
<td>DD 9.7</td>
<td>6.0</td>
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<tr>
<td>Abe → 3αMan</td>
<td>C-3</td>
<td>DD 7.4</td>
<td>5.5</td>
</tr>
</tbody>
</table>

Gal is D (from enzymatic oxidation) =>
Rha is L =>
Man is D =>
3,6ddXyl is D (=Abequose)

### Elucidated repeating unit

\[ \alpha-D-Galp(1\rightarrow3)\alpha-D-Manp(1\rightarrow4)\alpha-L-Rhap(1\rightarrow) \]
The C\textsuperscript{13}H spectrum of polysaccharide demonstrated a regular structure. It contained signals for four sugar residues, including those for four anomic carbons at 103.3, 102.8, 101.9 and 101.1, two unsubstttuted CH\textsubscript{2}OH groups, 15 oxygen-bearing sugar-ring carbons in the region 64-82, one CCH\textsubscript{3} group at 34.4 and two CH\textsubscript{2} groups at 18.6 and 17.0. Accordingly, the \textsuperscript{1}H NMR spectrum contained signals for four anomic protons at 5.34, 5.18, 5.10 and 5.06, signals of sugar-ring protons in region 3.5-4.2, one signal of a C-\textsubscript{CH}\textsubscript{2}C group at 2.00 and signals of two CH\textsubscript{2} groups at 1.34 and 1.19. As judged by the absence of signals within 82-88 region, all sugar residues are in pyranose form [1].

The sugar analysis of the polysaccharide revealed residues of Rha, Man and Gal residues in the ratio 1:1:1 and showed the presence of one more sugar, which has not been identified.

The \textsuperscript{1}H and \textsuperscript{13}C spectra of the polysaccharide were assigned using \{\textsuperscript{1}H, \textsuperscript{1}H\} COSY, TOCSY, ROESY, \{\textsuperscript{1}H, \textsuperscript{13}C\} HSQC, \{\textsuperscript{1}H, \textsuperscript{13}C\} HSQC-TOCSY and \{\textsuperscript{1}H, \textsuperscript{13}C\} HMBC experiments (Tables 1 and 2).

The C signals from C-1 to C-4 were assigned using the data of \{\textsuperscript{1}H, \textsuperscript{13}C\} HSQC experiment. Due to the strong overlap of signals from C-1 to C-4, the assignment was performed using the data of \{\textsuperscript{1}H, \textsuperscript{13}C\} HSQC-TOSY experiment, which contained the correlations of the following residues: Man-2/C-3 correlations at 72.9/78.6, 71.4/78.6, 71.0/78.6, 70.5/78.6; Gal-2/C-3 correlations at 72.0/78.6, 71.6/78.6; Rha-2/C-3 correlations at 72.0/78.6, 71.6/78.6; 3,6-deoxy-a-xylohexopyranoside [3].

The C signals from C-2 to C-6 of polysaccharide were assigned using the data of \{\textsuperscript{1}H, \textsuperscript{13}C\} HSQC-experiment. The signals for protons from H-2 to H-4 were assigned using the COSY spectrum, which showed all correlations between neighboring protons in this residue. However, as judged by \{\textsuperscript{1}H, \textsuperscript{13}C\} HSQC experiment, the signal for 3\textsubscript{d}6\textsubscript{d}Hex at 3.88 possessed a complete overlap with the signals of protons corresponding to C signals at 62.0 and 67.7. Due to this, the COSY experiment could be used for unambiguous assignment of the signal for 3\textsubscript{d}6\textsubscript{d}Hex.

The \textsuperscript{1}H and \textsuperscript{13}C NMR signals for Hex-II residue (Galp or Manp, accordingly to the sugar analysis, H-1 at 5.34) were assigned using the data of COSY, \{\textsuperscript{1}H, \textsuperscript{13}C\} HSQC, and \{\textsuperscript{1}H, \textsuperscript{13}C\} HMBC experiments. The signal for Hex-II H-2 was assigned basing on H-1/H-2 correlation at 4.04/5.11 in COSY spectrum. As there were no H-2/H-3 and H-3/H-4 correlations observed in COSY, the signals for H-3 and H-4 were assigned using the data of \{\textsuperscript{1}H, \textsuperscript{13}C\} HSQC and \{\textsuperscript{1}H, \textsuperscript{13}C\} HMBC experiments. Particularly, the HMBC spectrum demonstrated the intra-residue H-1/C-1=5.34/74.8, while chemical shifts for C-5 of unsubstituted a-Galp were distinguished basing on TOCSY spectrum that showed correlations of H-1/C-5 correlation at 69.7/4.12, and the H-5/H-6 correlation in COSY spectrum at 4.12/1.19 allowed to assign the signal for 3\textsubscript{d}6\textsubscript{d}Hex-2. The signals for C-5 and C-6 were assigned using the data of \{\textsuperscript{1}H, \textsuperscript{13}C\} HSQC experiment.

The spin system of Rha\textsubscript{p} was distinguished basing on TOCSY spectrum that showed correlations of Rha-6/H-4 (1.34) with all the other protons of Rha. The Rha \textsuperscript{1}H signal assignment was completed by the COSY spectrum, which contained all correlations between neighboring protons in this residue. The assignment of Rha \textsuperscript{13}C signals was performed using the data of \{\textsuperscript{1}H, \textsuperscript{13}C\} HSQC experiment and confirmed by \{\textsuperscript{1}H, \textsuperscript{13}C\} HSQC-TOCSY, which revealed the correlations of Rha-2 with all the carbon residues of the chemical shift for Rha-3 (69.4) indicated that this residue was in a-anomeric configuration [2]. The significant downfield displacement of Rha-4 signal (from 73.5 to 83.0) determined the substitution position as C-4.

The \textsuperscript{1}H and \textsuperscript{13}C chemical shifts of 3\textsubscript{d}6\textsubscript{d}Hex appeared to be characteristic for the terminal 3\textsubscript{d}6\textsubscript{d}-hexO-containing a-xylohexopyranoside [3].

The absolute configurations of residues: Gal for Man, L for Rha and D for 3,6-deoxy-xylohexopyranoside. 3,6-deoxy-D-xylohexopyranose is called "abequose". Thus the structure of repeating unit was elucidated as follows [slide 11].
**Tabular data**

**$^1$H NMR data (ppm) for the O-specific polysaccharide of *Citrobacter* PCM 1555**

<table>
<thead>
<tr>
<th></th>
<th>H-1</th>
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<th>H-3</th>
<th>H-4</th>
<th>H-5</th>
<th>H-6a</th>
<th>H-6b</th>
</tr>
</thead>
<tbody>
<tr>
<td>-Abe(1-)</td>
<td>5.11</td>
<td>4.04</td>
<td>2.00</td>
<td>3.88</td>
<td>4.12</td>
<td>1.19</td>
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<tr>
<td>2,3)-D-Manp(1)</td>
<td>5.34</td>
<td>4.02</td>
<td>4.05</td>
<td>3.87</td>
<td>~3.98</td>
<td>3.87</td>
<td>3.82</td>
</tr>
<tr>
<td>4)-L-Rhap(1)</td>
<td>5.06</td>
<td>4.07</td>
<td>~3.98</td>
<td>3.56</td>
<td>3.94</td>
<td>1.34</td>
<td></td>
</tr>
<tr>
<td>3)-D-Galp(1)</td>
<td>5.18</td>
<td>3.92</td>
<td>~3.95</td>
<td>4.07</td>
<td>4.10</td>
<td>3.75</td>
<td>3.69</td>
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</table>

**$^{13}$C NMR data (ppm) for the O-specific polysaccharide of *Citrobacter* PCM 1555**

<table>
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<td>72.9</td>
<td>62.5</td>
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</table>

**Referenced from “details”:**


**THIS WORK:**
Katzenellenbogen E, Kocharova NA, Toukach FV, Górska S, Korzeniowska-Kowal A, Bogulska M, Gamian A, Knirel YA
“Structure of an abequose-containing O-polysaccharide from *Citrobacter freundii* O22 strain PCM 1555”, *Carbohydr Res*, 2009, **344**(13), 1724-1728.